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(51) International Patent Classification ⁶: A61K 38/27	A1	(11) International Publication Number: WO 95/28173 (43) International Publication Date: 26 October 1995 (26.10.95)
(21) International Application Number: PCT/US95/03760 (22) International Filing Date: 30 March 1995 (30.03.95) (30) Priority Data: 08/228,548 15 April 1994 (15.04.94) US (71) Applicant: GENENTECH, INC. [US/US]; 460 Point San Bruno Boulevard, South San Francisco, CA 94080-4990 (US). (72) Inventors: BUNTING, Stuart; 1106 Date Street, Montara, CA 94037 (US). CLARK, Ross; 711 Ursula Avenue, Pacifica, CA 94044 (US). GILLETT, Nancy; 423 Palma, El Granada, CA 94018 (US). JIN, Hongkui; 622 Benvenue Avenue, Los Altos, CA 94022 (US). YANG, Renhui; 2045 Sea Cliff Way, San Bruno, CA 94066 (US). (74) Agents: HASAK, Janet, E. et al.; Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080-4990 (US).		(81) Designated States: AU, CA, JP, MX, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: TREATMENT OF CONGESTIVE HEART FAILURE (57) Abstract A mammal with congestive heart failure is treated by administering to the mammal an effective amount of growth hormone. Treatment results in increased left ventricular systolic pressure, increased left ventricular maximum, increased cardiac output, and increased stroke volume index. Treatment also results in reduced left ventricular end-diastolic pressure and reduced systemic vascular resistance. These measurements indicate improvement in cardiac function by increased ventricular contractility and decreased peripheral vascular resistance.		

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TREATMENT OF CONGESTIVE HEART FAILURE

BACKGROUND OF THE INVENTIONFIELD OF THE INVENTION

This invention relates to the field of treating patients having
5 congestive heart failure with growth hormone (GH).

DESCRIPTION OF BACKGROUND AND RELATED ART

In vitro studies have shown that chronic hypersecretion of growth hormone by implantation of a growth hormone-secreting tumor is associated with an increase in the maximum isometric force of left ventricular
10 papillary muscle normalized per cross-sectional area and no change in both the unloaded shortening velocity of the isolated muscle and the calcium and actin-activated myosin in normal rats. This is observed despite a marked shift of the isomyosin pattern toward the low ATPase activity V3 isoform. These results suggest that growth hormone may induce a unique pattern of
15 myocardial contraction: a normal shortening speed and an increased force generation are associated with changes in myosin phenotype that allow the cardiac muscle to function more economically. Timsit, J. et al., *J. Clin. Invest.* 86: 507-515 (1990); Timsit, J. et al., *Acta Paediatr Suppl* 383: 32-34 (1992).

20 In vitro studies from the same investigators were carried out on rat cardiac skinned fibers. These studies have demonstrated that the contractile performance of the skinned fiber from rat myocardium subjected to chronically high circulating growth hormone levels is increased. The increase in contractile performance was shown to be due to specific
25 alterations in the properties of the contractile apparatus, including an increase in both maximal tension and myofibrillar sensitivity to calcium. Mayoux, E. et al., *Circulation Research* 72(1): 57-64 (1993).

It has been found that left ventricular dp/dt , cardiac index, stroke index, and stroke work are significantly increased in chloralose-urethan-
30 anesthetized rats with a transplantable growth hormone-secreting tumor. The data suggest that chronic hypersecretion of growth hormone increases cardiac output by increasing contractility in anesthetized rats. Penney, D.G. et al., *Cardiovascular Research* 19: 270-277 (1985).

A contrary result has also been reported. Rubin, S.A. et al., *J.*
35 *Mol. Cell Cardiol.* 22: 429-438 (1990) have reported that similar chronic hypersecretion of growth hormone induced by the growth hormone-secreting

tumor causes significant decreases in left ventricular contractility (maximum dP/dt) and increases in LVEDP in ketamine-anesthetized rats.

In a clinical study, it has been shown that administration of human growth hormone to normal subjects for one week increases ventricular
5 contractility and cardiac output, as evaluated by echocardiography. Thuesen, L et. al., *Dan. Med. Bull.* 35: 193-196 (1988).

In adults with growth hormone deficiency, growth hormone treatment produced significant increases in stroke volume and exercise capacity. These results suggest that growth hormone can improve cardiac function at
10 rest and during exercise in the adult patients with growth hormone deficiency. Jorgensen, J. et. al., *Lancet* i: 1221-1225 (1989); Cuneo, R. et. al., *J. Appl. Physiol.* 70: 695-700 (1991); Christiansen, J. S. et. al., *Acta Paediatr Suppl* 383: 40-42 (1992).

Cuneo et. al., *Lancet* i:838-839 (1989) have reported a very
15 interesting case showing effects of growth hormone therapy in a patient with extremely poor cardiac function. The patient developed severe heart failure eight months after hypophysectomy for Cushing syndrome. Standard therapy including diuretics and angiotensin-converting enzyme inhibitors had little effect and cardiac transplantation was considered. As a last
20 resort, treatment with human growth hormone, 12 IU/day s.c. was tried, with a remarkable beneficial effect. Clinical improvement and increases in myocardial contractility and cardiac output were noted.

Until now, however, effects of human growth hormone in heart failure patients without growth hormone deficiency have not been reported, to
25 applicants' knowledge. Heart failure affects approximately three million Americans, developing in about 400,000 each year. Current therapy for heart failure is insufficient. Although angiotensin converting enzyme (ACE) inhibitors have been shown to have beneficial effects in patients with heart failure, they appear consistently unable to relieve symptoms in more
30 than 60% of heart failure patients. In addition, they reduce mortality of heart failure only by approximately 15-20%. Therefore, there is room for improvement in the therapy of heart failure.

Accordingly it is an object of this invention to provide a method of treatment for a patient with congestive heart failure.

35

SUMMARY

The present invention achieves this object by the provision of a method of treatment of congestive heart failure, the method characterized by administration of an effective amount of growth hormone (GH). The

administration of GH results in improvement of cardiac function by increased ventricular contractility and decreased peripheral vascular resistance.

BRIEF DESCRIPTION OF THE FIGURES

5 Figure 1a. Shows the body weight before treatment in ligated and sham controls. ** $P < 0.01$, compared to the respective vehicle group.

 Figure 1b. Shows an increase in body weight following treatment in ligated and sham controls. ** $P < 0.01$, compared to the respective vehicle group.

10 Figure 1c. Shows a comparison of the increase in the ratio of ventricular weight to body weight in ligated and sham controls. ** $P < 0.01$, compared to the respective vehicle group.

 Figure 2a. Shows the effect of GH administration on serum levels of GH in ligated and sham controls. ** $P < 0.01$, compared to the respective
15 vehicle group.

 Figure 2b. Shows the effect of GH administration on serum levels of IGF-1 in ligated and sham controls. ** $P < 0.01$, compared to the respective vehicle group.

 Figure 3a. Shows the effects of GH and vehicle on mean arterial pressure (MAP) in ligated rats and sham controls. # $P < 0.05$, ## $P < 0.01$, compared to the respective sham group. * $P < 0.05$, compared to the respective
20 vehicle group.

 Figure 3b. Shows the effects of GH on systolic arterial pressure (SAP) in ligated rats and sham controls. # $P < 0.05$, ## $P < 0.01$, compared to the respective sham group. * $P < 0.05$, compared to the respective vehicle
25 group.

 Figure 3c. Shows the effects of GH on heart rate (HR) in ligated rats and sham controls. # $P < 0.05$, ## $P < 0.01$, compared to the respective sham group. * $P < 0.05$, compared to the respective vehicle group.

30 Figure 4a. Shows the effects of GH on left ventricular maximum dP/dt. * $P < 0.05$, ** $P < 0.01$, compared to the respective vehicle group. # $P < 0.05$, ## $P < 0.01$, compared to the respective sham group.

 Figure 4b. Shows the effects of growth hormone (GH) on left ventricular systolic pressure (LVSP). * $P < 0.05$, ** $P < 0.01$, compared to the
35 respective vehicle group. # $P < 0.05$, ## $P < 0.01$, compared to the respective sham group.

 Figure 4c. Shows the effects of growth hormone (GH) on left ventricular end-diastolic pressure (LVEDP). * $P < 0.05$, ** $P < 0.01$, compared to

the respective vehicle group. # $P < 0.05$, ## $P < 0.01$, compared to the respective sham group.

Figure 5a. Shows the effects of growth hormone (GH) on cardiac index (CI) in ligated rats and sham controls. * $P < 0.05$, compared to the
5 respective vehicle group. # $P < 0.05$, compared to the respective sham group.

Figure 5b. Shows the effects of growth hormone (GH) on stroke volume index, (SVI) in ligated rats and sham controls. * $P < 0.05$, compared to the respective vehicle group. # $P < 0.05$, compared to the respective sham group.

10 Figure 5c. Shows the effects of growth hormone (GH) on systemic vascular resistance (SVR) in ligated rats and sham controls. * $P < 0.05$, compared to the respective vehicle group. # $P < 0.05$, compared to the respective sham group.

DETAILED DESCRIPTION OF THE INVENTION

15 a. Definitions

In general, the following words or phrases or abbreviations have the indicated definition when used in the description, examples, and claims:

As used herein, "BW" refers to body weight.

As used herein, "CO" refers to cardiac output.

20 As used herein, "CI" refers to cardiac index. The cardiac index is measurable as cardiac output divided by body weight (CO/BW).

As used herein, "dp/dt" refers to left ventricular maximum.

As used herein, "HR" refers to heart rate.

As used herein, "LCA" refers to left coronary artery.

25 As used herein, "LVEDP" refers to left ventricular end-diastolic pressure.

As used herein, "LVMP" refers to left ventricular mean pressure.

As used herein, "LVSP" refers to left ventricular systolic pressure.

As used herein, "MAP" refers to mean arterial pressure.

30 As used herein, "RAP" refers to right atrial pressure.

As used herein, "SAP" refers to systolic arterial pressure.

As used herein, "SV" refers to stroke volume. The stroke volume is measurable as CO/HR.

35 As used herein, "SVI" refers to stroke volume index. The stroke volume index is measurable as SV/BW.

As used herein, "SVR" refers to systemic vascular resistance. The SVR is measurable as MAP/CI.

As used herein, "VW" refers to ventricular weight.

As used herein "infarct" refers to an area of necrosis resulting from an insufficiency of blood supply. "Myocardial infarction" refers to myocardial necrosis resulting from the insufficiency of coronary blood supply.

As used herein "treatment" refers to ameliorating the congestive heart failure condition.

As used herein "congestive heart failure" refers to

As used herein, the term "mammal" refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal herein is human.

As used herein, "growth hormone" or "GH" refers to growth hormone in native-sequence or in variant form, and from any source, whether natural, synthetic, or recombinant. Examples include human growth hormone (hGH), which is natural or recombinant GH with the human native sequence (somatotropin or somatropin), and recombinant growth hormone (rGH), which refers to any GH or variant produced by means of recombinant DNA technology, including somatrem, somatotropin, and somatropin. Preferred herein for human use is recombinant human native-sequence, mature GH with or without a methionine at its N-terminus. More preferred is methionyl human growth hormone (met-hGH) produced in *E. coli*, e.g., by the process described in U.S. Pat. No. 4,755,465 issued July 5, 1988 and Goeddel et al., *Nature*, 282: 544 (1979). Met-hGH, which is sold under the trademark Protropin® by Genentech, Inc., is identical to the natural polypeptide, with the exception of the presence of an N-terminal methionine residue. This added amino acid is a result of the bacterial protein synthesis process. Also preferred is recombinant hGH available from Genentech, Inc. under the trademark Nutropin®. This latter hGH lacks this methionine residue and has an amino acid sequence identical to that of the natural hormone. See Gray et al., *Biotechnology*, 2: 161 (1984). Both methionyl hGH and hGH have equivalent potencies and pharmacokinetic values. Moore et al., *Endocrinology*, 122: 2920-2926 (1988). Another appropriate hGH candidate is an hGH variant that is a placental form of GH with pure somatogenic and no lactogenic activity as described in U.S. Pat. No. 4,670,393 issued 2 June 1987. Also included are GH variants as described in WO 90/04788 published 3 May 1990 and WO 92/09690 published 11 June 1992.

b. Modes for Carrying Out the InventionTherapeutic Compositions and Administration of GH

Therapeutic formulations of GH are prepared for storage by mixing GH having the desired degree of purity with optional physiologically acceptable carriers, excipients, or stabilizers (Remington's Pharmaceutical Sciences, supra), in the form of lyophilized cake or aqueous solutions. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronic or polyethylene glycol (PEG).

For administration, GH may be complexed or bound to a polymer to increase its circulatory half-life. Examples of polyethylene polyols and polyoxyethylene polyols useful for this purpose include polyoxyethylene glycerol, polyethylene glycol, polyoxyethylene sorbitol, polyoxyethylene glucose, or the like. The glycerol backbone of polyoxyethylene glycerol is the same backbone occurring in, for example, animals and humans in mono-, di-, and triglycerides.

The polymer need not have any particular molecular weight, but it is preferred that the molecular weight be between about 3500 and 100,000, more preferably between 5000 and 40,000. Preferably the PEG homopolymer is unsubstituted, but it may also be substituted at one end with an alkyl group. Preferably, the alkyl group is a C1-C4 alkyl group, and most preferably a methyl group. Most preferably, the polymer is an unsubstituted homopolymer of PEG, a monomethyl-substituted homopolymer of PEG (mPEG), or polyoxyethylene glycerol (POG) and has a molecular weight of about 5000 to 40,000.

The GH is covalently bonded via one or more of the amino acid residues of the GH to a terminal reactive group on the polymer, depending mainly on the reaction conditions, the molecular weight of the polymer, etc. The polymer with the reactive group(s) is designated herein as activated polymer. The reactive group selectively reacts with free amino

or other reactive groups on the GH. It will be understood, however, that the type and amount of the reactive group chosen, as well as the type of polymer employed, to obtain optimum results, will depend on the particular GH employed to avoid having the reactive group react with too many particularly active groups on the GH. As this may not be possible to avoid completely, it is recommended that generally from about 0.1 to 1000 moles, preferably 2 to 200 moles, of activated polymer per mole of protein, depending on protein concentration, is employed. The final amount of activated polymer per mole of protein is a balance to maintain optimum activity, while at the same time optimizing, if possible, the circulatory half-life of the protein.

While the residues may be any reactive amino acids on the protein, such as one or two cysteines or the N-terminal amino acid group, preferably the reactive amino acid is lysine, which is linked to the reactive group of the activated polymer through its free epsilon-amino group, or glutamic or aspartic acid, which is linked to the polymer through an amide bond.

The covalent modification reaction may take place by any appropriate method generally used for reacting biologically active materials with inert polymers, preferably at about pH 5-9, more preferably 7-9 if the reactive groups on the GH are lysine groups. Generally, the process involves preparing an activated polymer (with at least one terminal hydroxyl group), preparing an active substrate from this polymer, and thereafter reacting the GH with the active substrate to produce the GH suitable for formulation. The above modification reaction can be performed by several methods, which may involve one or more steps. Examples of modifying agents that can be used to produce the activated polymer in a one-step reaction include cyanuric acid chloride (2,4,6-trichloro-S-triazine) and cyanuric acid fluoride.

In one embodiment the modification reaction takes place in two steps wherein the polymer is reacted first with an acid anhydride such as succinic or glutaric anhydride to form a carboxylic acid, and the carboxylic acid is then reacted with a compound capable of reacting with the carboxylic acid to form an activated polymer with a reactive ester group that is capable of reacting with the GH. Examples of such compounds include N-hydroxysuccinimide, 4-hydroxy-3-nitrobenzene sulfonic acid, and the like, and preferably N-hydroxysuccinimide or 4-hydroxy-3-nitrobenzene sulfonic acid is used. For example, monomethyl substituted PEG may be reacted at elevated temperatures, preferably about 100-110°C for four

hours, with glutaric anhydride. The monomethyl PEG-glutaric acid thus produced is then reacted with N-hydroxysuccinimide in the presence of a carbodiimide reagent such as dicyclohexyl or isopropyl carbodiimide to produce the activated polymer, methoxypolyethylene glycolyl-N-succinimidyl glutarate, which can then be reacted with the GH. This method is described in detail in Abuchowski et al., Cancer Biochem. Biophys., 7: 175-186 (1984). In another example, the monomethyl substituted PEG may be reacted with glutaric anhydride followed by reaction with 4-hydroxy-3-nitrobenzene sulfonic acid (HNSA) in the presence of dicyclohexyl carbodiimide to produce the activated polymer. HNSA is described by Bhatnagar et al., Peptides: Synthesis-Structure-Function. Proceedings of the Seventh American Peptide Symposium, Rich et al. (eds.) (Pierce Chemical Co., Rockford IL, 1981), p. 97-100, and in Nitecki et al., High-Technology Route to Virus Vaccines (American Society for Microbiology: 1986) entitled "Novel Agent for Coupling Synthetic Peptides to Carriers and Its Applications."

Specific methods of producing GH conjugated to PEG include the methods described in U.S. Pat. No. 4,179,337 on PEG-GH and U.S. Pat. No. 4,935,465, which discloses PEG reversibly but covalently linked to GH. Other specific methods for producing PEG-GH include the following:

PEGylation with methoxypolyethylene glycol aldehyde (Me-PEG aldehyde) by reductive alkylation and purification is accomplished by adding to 2 mg/mL of GH in PBS pH 7.0, 5 mM of Me-PEG aldehyde-5000 (molecular weight 5000 daltons) and 20 mM of NaCNBH₃ and gently mixing at room temperature for 3 hours. Ethanolamine is then added to 50 mM to reductively amidate the remaining unreacted Me-PEG. The mixture is separated on an anion-exchange column, FPLC Mono Q. The surplus unreacted Me-PEG does not bind to the column and can then be separated from the mixture. Two main PEGylated GH fractions are obtained with apparent molecular weights of 30K and 40K on reduced SDS-PAGE, vs. 20K of the unreacted GH. GH-GHBP complex is PEGylated in the same manner to give a derivative of 150K by gel filtration.

PEGylation with N-hydroxysuccinimidyl PEG (NHS-PEG) and purification are accomplished by adding NHS-PEG at a 5-fold molar excess of the total lysine concentration of GH to a solution containing 2 mg/mL of GH in 50 mM of sodium borate buffer at pH 8.5 or PBS at pH 7, and mixing at room temperature for one hour. Products are separated on a Superose 12 sizing column and/or Mono Q of FPLC. The PEGylated GH varies in size depending on the pH of the reaction from approximately 300 K for the reaction run at

pH 8.5 to 40 K for pH 7.0 as measured by gel filtration. The GH-GHBP complex is also PEGylated the same way with a resulting molecular weight of 400 to 600 Kd from gel filtration.

PEGylation of the cysteine mutants of GH with PEG-maleimide is accomplished by preparing a single cysteine mutant of GH by site-directed mutagenesis, secreting it from an *E. coli* 16C9 strain (W3110 Δ tonA phoA Δ E15 Δ (argF-lac)169 deoC2 that does not produce the deoC protein), and purifying it on an anion-exchange column.

Strain 16C9 was constructed genetically by transferring the deoC2 allele from strain CGSC#6092 (No. 6092, available from the *E. coli* Genetic Stock Center, New Haven, Conn. and described in Mark et al., Molec. Gen. Genet., 155: 145-152 (1977), with genotype *trxA1 recA1 ilvE720::tn5 metE70 deoC2 lacZ53 rha5 malB45 rpsL151*) into a strain designated 7C1.

Strain 7C1 [with genotype W3110 Δ tonA phoA Δ E15 Δ (argF-lac)169] was constructed in several steps using techniques involving transductions with phage P1Kc, derived from P1 (J. Miller, Experiments in Molecular Genetics [Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory, 1972]), and transposon genetics (Kleckner et al., J. Mol. Biol., 116: 125-159 [1977]). *E. coli* K12 W3110, which is a K12 strain that is F-, 8- (the wild type is F+, 8+) (Bachmann, Bact. Rev., 36: 525-557 [1972]), was used as the starting host.

First, the *tonA* gene (*fhuA*) (Kadner et al., J. Bact., 143: 256-264 [1980]; Bachmann, Microbiol. Rev., 47: 180-230 [1983]) was deleted by the insertion and subsequent imprecise excision of a Tn10 transposon into the *tonA* gene.

In the first step of this procedure, *E. coli* W3110 was transduced with λ ::Tn10 to generate a Tn10 hop pool of *E. coli* W3110 (Kleckner et al., J. Mol. Biol., *supra*).

The *E. coli* W3110::Tn10 hop pool was grown in L broth at 37°C to a cell density of about 1×10^9 /mL. A total of 0.5 mL of the culture was centrifuged and the pellet was resuspended in 0.2 mL of a 8phi80 lysate containing 7.0×10^9 pfu. The phage was allowed to adsorb for 30 minutes at 37°C. The suspension was then spread on EMB plates supplemented with tetracycline (15 μ g/mL). After an overnight incubation at 37°C, the colonies were pooled in 3 mL of L broth, grown overnight at 37°C, washed twice, and resuspended in L broth. A bacteriophage P1Kc lysate was made on this culture (Miller, J.H., Experiments in Molecular Biology, *supra*, page 304).

E. coli AT982 (no. 4546, *E. coli* Genetic Stock Center, New Haven, Conn.) was transduced to tetracycline resistance by this Plkc lysate. Transductants were selected on L broth plates supplemented with tetracycline (15 µg/mL) and 40 µg/mL diaminopimelic acid (dap). The
5 resulting transductants were screened for tetracycline resistance and the regeneration of the *dap* gene (*dap*⁺, *tet*^R). *dap*⁺, *tet*^R transductants were then tested for *λ*phi80 resistance.

Plkc lysates were then made on several *dap*⁺, *tet*^R, *λ*phi80-resistant strains. The lysates were used to transduce *E. coli* W3110 to tetracycline
10 resistance. The transductants were screened and selected for *λ*phi80 resistance.

Tetracycline-sensitive isolates were selected from the W3110 *tonA::Tn10-λ*phi80R transductants. Maloy and Nunn, *J. Bacteriol.*, 145: 1110 (1981). These isolates were checked for *λ*phi80 resistance and tetracycline
15 sensitivity after single colony purification.

DNA was isolated from several tetracycline-sensitive *λ*phi80-resistant mutants and digested with *Sst*II. The *Sst*II-digested DNA was characterized by the Southern blot procedure using radioactively labeled and *Sst*II-digested *λ::Tn10* DNA as a probe to determine if the *Tn10* had been excised.
20 Davis et al., *Advanced Bacterial Genetics* (Cold Spring Harbor Laboratory, New York, 1980). One of the tetracycline-sensitive isolates was shown to have lost two of the *Tn10* hybridization bands as compared to the hybridization between DNA from the *λ::Tn10* and the parental W3110 *tonA::Tn10λ*phi80R. A third hybridization band had an altered mobility,
25 indicating that a deletion caused by the imprecise excision of *Tn10* had occurred.

SDS-gel electrophoresis of outer membrane preparations from the strain with an imprecise *Tn10* excision revealed that the band assumed to be the TonA protein had an altered electrophoretic mobility as compared to
30 the wild-type TonA protein. The resulting protein was non-functional as a *λ*phi80 phage receptor protein. A second independent strain that also had undergone imprecise excision of *Tn10* showed no TonA protein on the SDS gel.

Neither of these strains demonstrated reversion to tetracycline resistance or to *λ*phi80 susceptibility, indicating that there was an
35 imprecise excision of all or part of the *Tn10* transposon together with either a partial or complete deletion of the *tonA* gene. Thus, the TonA protein (MW 78,000) was eliminated from the outer membrane, rendering the W3110 *tonA* strain resistant to several bacteriophages.

Then, two more deletion mutations, *phoA* Δ E15 (Sarchy et al., J. Bact., 145: 288-292 [1981]) and) (*argF-lac*)-169 (Schweizer et al., Mol. Gen. Genet., 192: 293-294 [1983]), were simultaneously transferred into W3110 *tonA* by genetic linkage to a kanamycin-resistance transposon inserted
5 into a proline biosynthetic gene (*proC::Tn5*).

The transposon was eliminated by selecting for a spontaneous prototrophic (*pro*⁺) revertant on glucose minimal agar plates. The introduction of the *phoA* mutation was recognized as transductants that form white colonies on glucose-minimal agar plates with 0.2 mM phosphate and 20
10 mg/L 5-bromo-4-chloro-3-indolyl phosphate. Likewise, the) (*argF-lac*)-169 mutation causes the loss of the enzyme beta-galactosidase and results in cells that form white colonies on MacConkey-1% lactose agar plates. The result was strain 7C1.

Finally, the *deoC* mutation (Bachmann, *supra*), removing the aldolase,
15 was introduced into 7C1 by a multistep process of transductions using phage *Plkc*. Standard methods for transduction were utilized. First, threonine auxotrophy was introduced into 7C1 to provide a means for positive selection of transduced chromosomal segments in the region of the *deoC* gene as follows.

20 *Plkc* was grown on a threonine auxotroph, such auxotrophs being described in Clare N. Berg and Douglas E. Berg, Microbiology-1981, "Bacterial Transposons", pp. 107-116 (Amer. Soc. for Microbiology, Washington, DC, 1981).

The resulting lysate was used to transduce strain 7C1 to tetracycline
25 resistance, selecting for transductants on LB plates containing 25 μ g/mL tetracycline. The resulting strain, designated 14A9 (*tonA* Δ , *phoA* Δ E15, Δ (*argF-lac*)-169 *thr::tn10*), reverted spontaneously to prototrophy at a high frequency, so fusaric acid plates (J. Bact., 145: 1110 [1981]) were used to select a stable tetracycline-sensitive threonine auxotroph, designated
30 strain 16C4.

Plkc was grown on Strain CGSC#6092, described *supra*.

The resulting lysate was used to transduce strain 16C4 to prototrophy, selecting for growth on glucose minimal agar plates. To obtain a high-frequency transducing lysate from strain 2D4, the *Plkc* phage
35 had to be cycled for growth two times on this host. Five prototrophic transductants of strain 16C4 were isolated, purified, and tested for growth on thymidine minimal agar plates. Four out of five of these isolates could not grow on thymidine and therefore had received the *deoC2* mutation that

eliminates synthesis of the deoC protein. One of these four isolates was saved and was designated strain 16C9 (Δ tonA, phoA, Δ E15, Δ (argF-lac)169, deoC2).

PEG-maleimide is made by reacting monomethoxyPEG amine with sulfo-MBs
5 in 0.1 M sodium phosphate pH 7.5 for one hour at room temperature and buffer exchanged to phosphate buffer pH 6.2. Next GH with a free extra cysteine is mixed in for one hour and the final mixture is separated on a Mono Q column as in Me-PEG aldehyde PEGylated GH.

As ester bonds are chemically and physiologically labile, it may be
10 preferable to use a PEG reagent in the conjugating reaction that does not contain ester functionality. For example, a carbamate linkage can be made by reacting PEG-monomethyl ether with phosgene to give the PEG-chloroformate. This reagent could then be used in the same manner as the NHS ester to functionalize lysine side-chain amines. In another example,
15 a urea linkage is made by reacting an amino-PEG-monomethyl ether with phosgene. This would produce a PEG-isocyanate that will react with lysine amines.

A preferred manner of making PEG-GH, which does not contain a cleavable ester in the PEG reagent, is described as follows:
20 Methoxypoly(ethylene glycol) is converted to a carboxylic acid by titration with sodium naphthalene to generate the alkoxide, followed by treatment with bromoethyl acetate to form the ethyl ester, followed by hydrolysis to the corresponding carboxylic acid by treatment with sodium hydroxide and water, as reported by Bückmann et al., Macromol. Chem., 182: 1379-1384
25 (1981). The resultant carboxylic acid is then converted to a PEG-N-hydroxysuccinimidyl ester suitable for acylation of GH by reaction of the resultant carboxylic acid with dicyclohexylcarbodiimide and NHS in ethyl acetate.

The resultant NHS-PEG reagent is then reacted with 12 mg/mL of GH
30 using a 30-fold molar excess over GH in a sodium borate buffer, pH 8.5, at room temperature for one hour and applied to a Q Sepharose column in Tris buffer and eluted with a salt gradient. Then it is applied to a second column (phenyl Toyopearl) equilibrated in 0.3 M sodium citrate buffer, pH 7.8. The PEGylated GH is then eluted with a reverse salt gradient, pooled,
35 and buffer-exchanged using a G25 desalting column into a mannitol, glycine, and sodium phosphate buffer at pH 7.4 to obtain a suitable formulated PEG7-GH.

The PEGylated GH molecules and GH-GHBP complex can be characterized by SDS-PAGE, gel filtration, NMR, tryptic mapping, liquid chromatography-mass spectrophotometry, and in vitro biological assay. The extent of PEGylation is suitably first shown by SDS-PAGE and gel filtration and then
5 analyzed by NMR, which has a specific resonance peak for the methylene hydrogens of PEG. The number of PEG groups on each molecule can be calculated from the NMR spectrum or mass spectrometry. Polyacrylamide gel electrophoresis in 10% SDS is appropriately run in 10 mM Tris-HCl pH 8.0, 100 mM NaCl as elution buffer. To demonstrate which residue is PEGylated,
10 tryptic mapping can be performed. Thus, PEGylated GH is digested with trypsin at the protein/enzyme ratio of 100 to 1 in mg basis at 37°C for 4 hours in 100 mM sodium acetate, 10 mM Tris-HCl, 1 mM calcium chloride, pH 8.3, and acidified to pH < 4 to stop digestion before separating on HPLC Nucleosil C-18 (4.6 mm X 150 mm, 5 μ ,100A). The chromatogram is compared
15 to that of non-PEGylated starting material. Each peak can then be analyzed by mass spectrometry to verify the size of the fragment in the peak. The fragment(s) that carried PEG groups are usually not retained on the HPLC column after injection and disappear from the chromatograph. Such disappearance from the chromatograph is an indication of PEGylation on that
20 particular fragment that should contain at least one lysine residue. PEGylated GH may then be assayed for its ability to bind to the GHBP by conventional methods.

The various PEGylation methods used produced various kinds of PEGylated wild-type GH, with apparent molecular weights of 35K, 51K, 250K,
25 and 300K by size exclusion chromatography, which should be close to their native hydrodynamic volume. These were designated PEG1-GH, PEG2-GH, PEG3-GH, and PEG7-GH, respectively. From the results of the tryptic mapping, the PEG1-GH and PEG2-GH both had the N-terminal 9-amino-acid fragment missing from the chromatogram and possibly PEGylated, which could be
30 confirmed by the mass spectrometry of the big molecular species found in the flow-through of the liquid chromatograph. From the molecular weight on SDS-PAGE, PEG1-GH may have one PEG on the N-terminal amine, and the PEG2-GH may have two PEG molecules on the N-terminal amine, forming a tertiary amide. The PEG3-GH has about 5 PEG groups per molecule based upon
35 the NMR result, and on the tryptic map, at least five peptide fragments were missing, suggesting that they are PEGylated. The PEG7-GH molecule is believed to have 6-7 PEG groups per molecule based on mass spectrometry.

The sites for adding PEG groups to GH, and those that are preferred residues for such conjugation, are N-terminal methionine or phenylalanine, lysine 38, lysine 41, lysine 70, lysine 140, lysine 145, lysine 158, and lysine 168. Two lysines that appeared not to be PEGylated were lysine 115
5 and lysine 172.

The GH to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution. The GH ordinarily will be stored in lyophilized form or in solution.

10 Therapeutic GH compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

The route of GH administration is in accord with known methods. Examples of parenteral administration include subcutaneous, intramuscular,
15 intravenous, intraarterial, and intraperitoneal administration, or by sustained release systems as noted below. Subcutaneous and intravenous injection or infusion is preferred.

Suitable examples of sustained-release preparations include semipermeable polymer matrices in the form of shaped articles, e.g. films,
20 or microcapsules. Sustained release matrices include polyesters, hydrogels, polylactides (U.S. 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman et al., Biopolymers 22:547-556 [1983]), poly (2-hydroxyethyl-methacrylate) (Langer et al., J. Biomed. Mater. Res. 15:167-277 [1981] and Langer, Chem. Tech. 12:98-105
25 [1982]), ethylene vinyl acetate (Langer et al., supra) or poly-D-(-)-3-hydroxybutyric acid (EP 133,988). Sustained-release GH compositions also include liposomally entrapped GH. Liposomes containing GH are prepared by methods known per se: DE 3,218,121; Epstein et al., Proc. Natl. Acad. Sci. USA 82:3688-3692 (1985); Hwang et al., Proc. Natl. Acad. Sci. USA 77:4030-
30 4034 (1980); EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese patent application 83-118008; U.S. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. % cholesterol, the selected proportion being adjusted for the
35 optimal GH therapy.

An "effective amount" of GH to be employed therapeutically will depend, for example, upon the route of administration, and the condition of the patient. Accordingly, it will be necessary for the therapist to

titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. Typically, the clinician will administer the GH until a dosage is reached that achieves the desired effect. The progress of this therapy is easily monitored by conventional assays.

In the treatment of congestive heart failure by GH, the GH composition will be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular mammal being treated, the clinical condition of the individual patient, the site of delivery of the GH, the particular type of GH, the method of administration, the scheduling of administration, and other factors known to medical practitioners. The "therapeutically effective amount" of GH to be administered will be governed by such considerations, and is the minimum amount necessary to ameliorate, or treat the congestive heart failure, so as to increase ventricular contractility and decrease peripheral vascular resistance, or to ameliorate other conditions of similar importance in congestive heart failure patients. Such amount is preferably below the amount that is toxic to the host or renders the host significantly more susceptible to infections.

As a general proposition, the total pharmaceutically effective amount of GH administered parenterally per dose will be in the range of about 1 μ g/kg/day to 10 mg/kg/day of patient body weight, although, as noted above, this will be subject to a great deal of therapeutic discretion. More preferably, this dose is at least 0.01 mg/kg/day, and most preferably for humans between about 0.01 and 1 mg/kg/day. If given continuously, the GH is typically administered at a dose rate of about 1 μ g/kg/hour to about 50 μ g/kg/hour, either by 1-4 injections per day or by continuous subcutaneous infusions, for example, using a mini-pump. An intravenous bag solution may also be employed. Preferably, in human patients, a pharmaceutically effective amount of the GH administered parenterally per dose will be in the range of about 10 to 100 micrograms per kilogram of patient body weight per day.

It is noted that practitioners devising doses of both IGF-I and GH should take into account the known side effects of treatment with this hormones.

As noted above, however, these suggested amounts of GH are subject to a great deal of therapeutic discretion. The key factor in selecting an appropriate dose and scheduling is the result obtained, as indicated above.

EXAMPLES

Use of GH to Treat Congestive Heart Failure

Introduction

The goal of this study was to examine the cardiac effects of human
5 GH treatment in an animal model of congestive heart failure.

Methods

Male Sprague-Dawley (SD) rats (Charles River Breeding Laboratories,
Inc., eight weeks of age) were acclimated to the facility for at least 1
week before surgery. Rats were fed a pelleted rat chow and water ad
10 libitum and housed in a light and temperature controlled room.

Coronary Arterial ligation

Myocardial infarction was produced by left coronary arterial ligation as
described previously. Greenen, D.L. et al., *J. Appl. Physiol.* 63: 92-96
(1987); Buttrick, P. et al., *Am. J. Physiol.* 260: 11473-11479 (1991). The
15 rats were anesthetized with sodium pentobarbital (60) mg/kg, ip), intubated
via tracheotomy, and ventilated by a respirator (Harvard Apparatus Model
683). After a left-sided thoracotomy, the left coronary artery was ligated
approximately 2 mm from its origin with a 7-0 silk suture. Sham animals
underwent the same procedure except that the suture was passed under the
20 coronary artery and then removed. All rats were handled according to the
"Position of the American Heart Association on Research Animal Use" adopted
November 11, 1984 by the American Heart Association. 4-6 weeks after
ligation myocardial infarction could develop heart failure in rats. In
clinical patients, myocardial infarction or coronary artery disease is the
25 most common cause of heart failure. Congestive heart failure in this model
reasonably mimics congestive heart failure in most human patients.

Electrocardiograms

One week after surgery, electrocardiograms were obtained under light
metofane anesthesia to document the development of infarcts. The ligated
30 rats of this study were subgrouped according to the depth and persistence
of pathological Q waves across the precordial leads Buttrick, P. et al.,
Am. J. Physiol. 260: 11473-11479 (1991); Kloner, R.A. et al., *Am Heart J.*
51: 1009-1013 (1983). This provided a gross estimate of infarct size and
assured that large and small infarcts were not differently distributed in
35 the ligated rats treated with GH and vehicle. Confirmation was made by
precise infarct size measurement.

GH Administration

Four weeks after surgery, recombinant human GH (1 mg/kg twice a day for 15 days) (Genentech, Inc., South San Francisco, CA) or saline vehicle was injected subcutaneously in both ligated rats and sham controls. Previous studies have shown that this dose of human GH can produce significant anabolic effects in rats. Moore et al., *Endocrinology* 122: 2920-2926 (1988); R. Clark and M. Cronin, U.S. Patent No. 5, 126,324 (1992). Body weight (BW) was measured twice a week during the treatment. See Figure 1. GH can be administered in saline or water as vehicles.

Catheterization

After 13 day treatment of GH or vehicle, rats were anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneal). A catheter (PE-10 fused with PE 50) filled with heparin-saline solution (50 U/ml) was implanted into the abdominal aorta through the right femoral artery for measurement of arterial pressure and heart rate. A second catheter (PE 50) was implanted into the right atrium through the right jugular vein for measurement of right atrial pressure and for saline injection. For measurement of left ventricular pressures and contractility (dP/dt), a third catheter (PE 50) was implanted into the left ventricle through the right carotid artery. For the measurement of cardiac output by a thermodilution method, a thermistor catheter (Lyons Medical Instrument Co., Sylmar, CA) was inserted into the aortic root. The catheters were exteriorized at the back of the neck with the aid of a stainless steel wire tunneled subcutaneously and then fixed. Following catheter implantation, all rats were housed individually.

Hemodynamic measurements

One day after catheterization, the thermistor catheter was processed in a microcomputer system (Lyons Medical Instrument Co.) for cardiac output determination, and the other three catheters were connected to a Model CP-10 pressure transducer (Century Technology Company, Inglewood, CA, USA) coupled to a Grass Model 7 polygraph (Grass Instruments, Quincy, MA, USA). Mean arterial pressure (MAP), systolic arterial pressure (SAP), heart rate (HR), right atrial pressure (RAP), left ventricular systolic pressure (LVSP), left ventricular mean pressure (LVMP), left ventricular end-diastolic pressure (LVEDP) and left ventricular maximum (dP/dt) were measured in conscious, unrestrained rats. For measurement of cardiac output, 0.1 ml of isotonic saline at room temperature was injected as a bolus via the jugular vein catheter. The thermodilution curve was monitored by VR-16 simultrace recorders (Honeywell Co., NY) and cardiac

output (CO) was digitally obtained by the microcomputer. Stroke volume (SV)=CO/HR; Cardiac index (CI)=CO/BW; Systemic vascular resistance (SVR)=MAP/CI.

After measurement of these hemodynamic parameters, 1 ml blood was collected through the arterial catheter. Serum was separated and stored at - 70°C for measurement of GH and IGF-1.

At the conclusion of the experiments, rats were anesthetized with pentobarbital sodium (60 mg/kg) and the heart was arrested in diastole with intra-atrial injection of KCl (1 M). The heart was removed, and the atria and great vessels were trimmed from the ventricle. The ventricle was weighed and fixed in 10% buffered formalin. See Figure 1, Bottom.

All experimental procedures were approved by Genentech's Institutional Animal Care and Use Committee before initiation of the study.

Infarct size measurements

The right ventricular free wall was dissected from the left ventricle. The left ventricle was cut in four transverse slices from apex to base. Five micrometer sections were cut and stained with Masson's trichrome stain and mounted. The endocardial and epicardial circumferences of the infarcted and non-infarcted left ventricular were determined with a planimeter Digital Image Analyzer. The infarcted circumference and the left ventricular circumference of all four slices were summed separately for each of the epicardial and endocardial surfaces and the sums were expressed as a ratio of infarcted circumference to left ventricular circumference for each surface. These two ratios were then averaged and expressed as a percentage for infarct size.

Hormone assays

Serum human GH was measured by a sensitive ELISA. A. Celniker (abstr) *Am. Endocrin Soc.*, A. Celniker et al., *Endocrinol. Metab.* 68(2):469 (1989); Greenen, D.L. et al., *J. Appl. Physiol.* 63: 92-96 (1987). This assay does not detect rat GH. Total serum IGF-1 was measured after acid-ethanol extraction by radioimmunoassay, for example, RIA described by Furlanetto et al., *J. Clin. Invest.* 60: 648-657 (1977); Bala and Bhaumick, *J. Clin. Endocrinol. and Metab.* 49: 770-777 (1979); Zapf et al., *J. Clin. Invest.* 68: 1321-1330 (1981); Hall et al., *J. Clin. Endo. Metab.* 48: 271-278 (1979); EP 292,656., using human IGF-1 (Genentech M3-RD1) as the standard and a rabbit anti-IGF-1 polyclonal antiserum. The acceptable range was 1.25-40 ng/ml, while the intra and inter-assay variability were 5-9% and 6-15%, respectively. See Figure 2.

Statistical analysis

Results are expressed as mean \pm SEM. Two way and one way analysis of variance was performed to assess differences in parameters between groups. Significant differences were then subjected to post hoc analysis using the Newman-Keuls method. $P < 0.05$ was considered significant.

Results

The mean BW before treatment of GH or vehicle was not different between the experimental groups (Figure 1A). There was significantly greater increase in BW following GH treatment for both sham and ligated rats (Figure 1B). LCA ligation caused a significant increase in the ratio of ventricular weight (VW) to BW, while GH treatment did not alter this ratio significantly (Figure 1C).

GH treatment significantly increased serum levels of human GH and IGF-1 in both sham ligated rats (Figures 2A and 2B). The GH-induced increment in serum levels of human GH and IGF-1 was not significantly different between sham and ligated rats.

Infarct size in ligated rats was not different between the vehicle-treated group ($33.2 \pm 2.2\%$ of the left ventricle) and the GH-treated group ($31.4 \pm 2.6\%$ of the left ventricle).

LCA ligation resulted in significant decreases in MAP in the vehicle-treated rats but not in the GH-treated rats (Figure 3A). GH treatment significantly decreased MAP in the sham rats but not in the ligated rats. LCA ligation was associated with significant reductions in SAP in both vehicle-treated and GH-treated rats (Figure 3B). However, the ligation-induced decrease in SAP was significantly greater in the vehicle-treated rats than that in the GH-treated rats. GH administration did not alter SAP significantly in the sham rats. Neither LCA ligation nor GH treatment altered HR significantly (Figure 3C).

LCA ligation significantly lowered left ventricular dP/dt and LVSP in the vehicle-treated rats but not in the GH-treated rats (Figures 4A and B). GH treatment increased dP/dt and LVSP in the ligated rats but not in the sham rats.

In the vehicle-treated animals, LVEDP was significantly elevated in the ligated group compared to sham controls (Figure 4C). In the GH-treated animals, however, there was no significant difference in LVEDP between the ligated and sham group. GH administration decreased LVEDP significantly in the ligated rats but not in the sham rats.

LCA ligation produced significant reductions in CI and SVI in the vehicle treated rats but not in the GH-treated rats (Figure 5A and B). GH administration significantly increased CI and SVI in the ligated rats but not in sham controls. There was no significant difference in SVR between
5 the ligated and sham rats, while GH treatment significantly lowered SVR in the ligated rats and tended to lower SVR in sham controls (Figure 5C).

In the current study, 6 weeks after left coronary artery (LCA) ligation, rats treated with vehicle exhibited significant decreases in LVSP, dP/dt, CI, and SVI and increases in LVEDP compared to the sham
10 controls. These results indicate that congestive heart failure occurred in this animal model of myocardial infarction primarily due to a decrease in ventricular contractility. GH treatment at the dose of 2 mg/kg/day for 15 days significantly increased LVSP, dP/dt, CO, and SVI and reduced LVEDP and SVR in the LCA ligated rats. This result demonstrates that administration
15 of GH improves cardiac function by increasing ventricular contractility and decreasing peripheral vascular resistance in congestive heart failure. In sham rats, however, GH administration at this dose did not significantly alter cardiac function except slightly lowering arterial pressure and peripheral vascular resistance.

20 It would be reasonably expected that the rat data herein may be extrapolated to horses, cows, humans and other mammals, correcting for the body weight of the mammal in accordance with recognized veterinary and clinical procedures. Using standard protocols and procedures, the veterinarian or clinician will be able to adjust the doses, scheduling, and
25 mode of administration of GH and its variants to achieve maximal effects in the desired mammal being treated. Humans are believed to respond in this manner as well.

WE CLAIM:

1. A method of treating a mammal exhibiting congestive heart failure comprising administering to said mammal an effective amount of growth hormone.
- 5 2. The method of claim 1 wherein said growth hormone is human growth hormone.
3. The method of claim 1 wherein said mammal is human.
4. The method of claim 3 wherein said effective amount is in the range of 10-100 micrograms per kilogram of body weight per day.
- 10 5. The method of claim 3 wherein said administering is subcutaneous or intravenous.
6. The method of claim 1 wherein said congestive heart failure results from myocardial infarction.

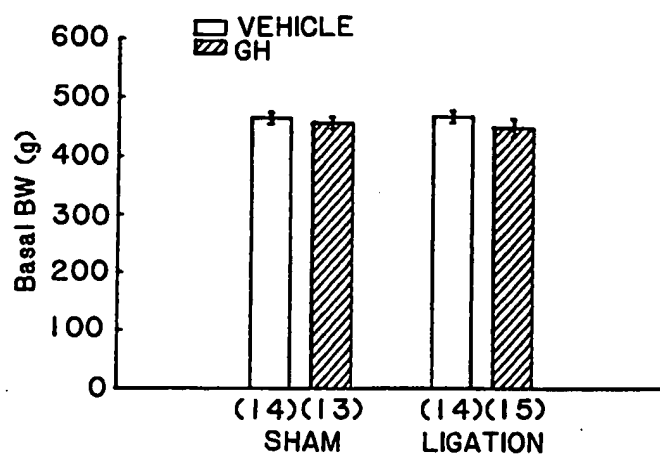


FIG. 1a

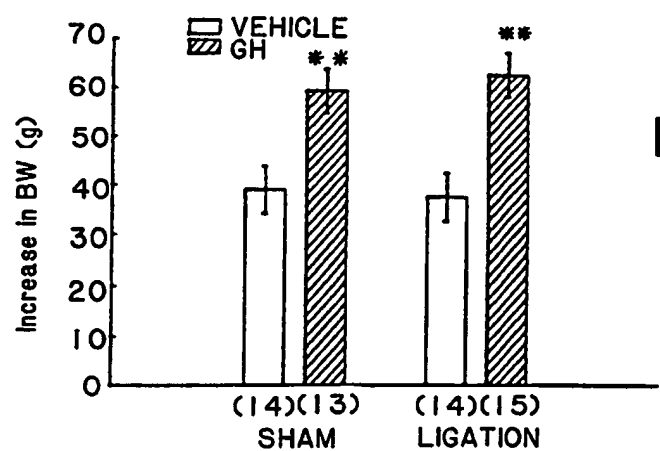


FIG. 1b

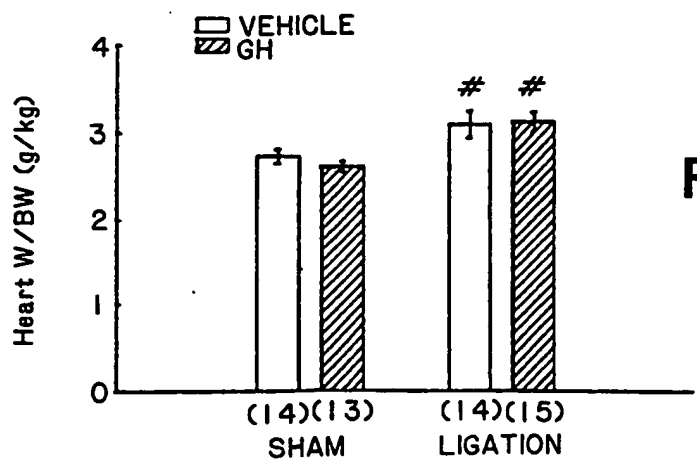


FIG. 1c

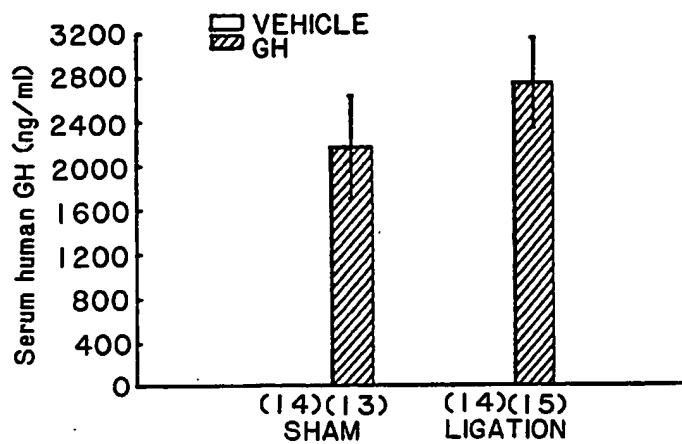


FIG. 2a

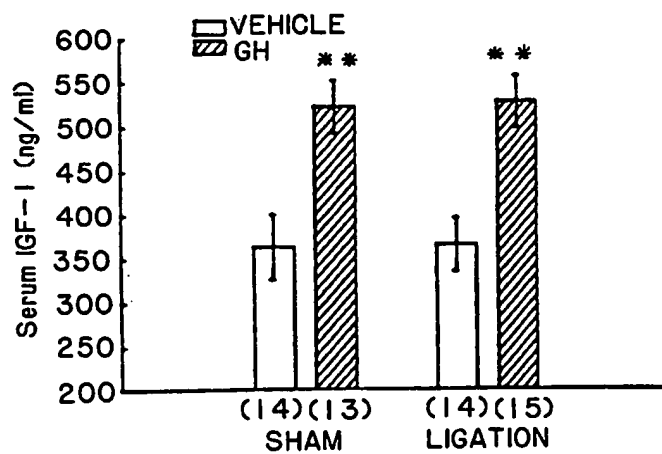


FIG. 2b

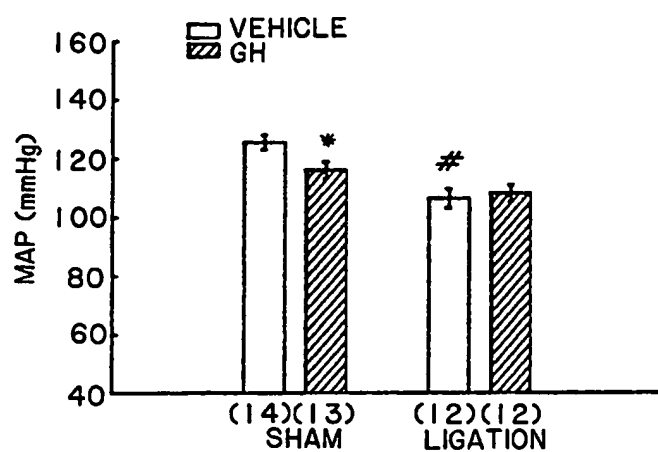


FIG. 3a

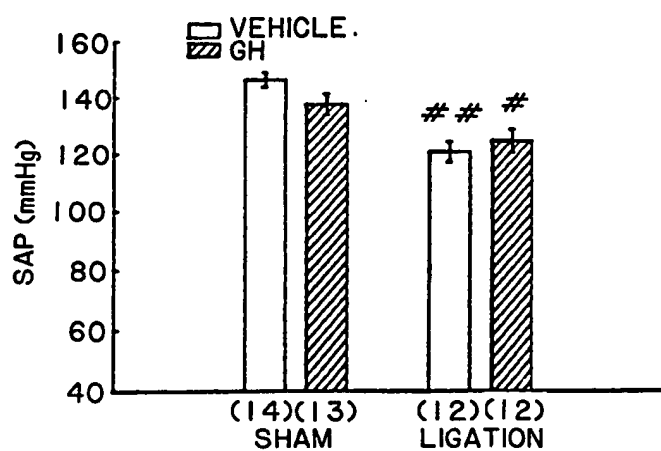


FIG. 3b

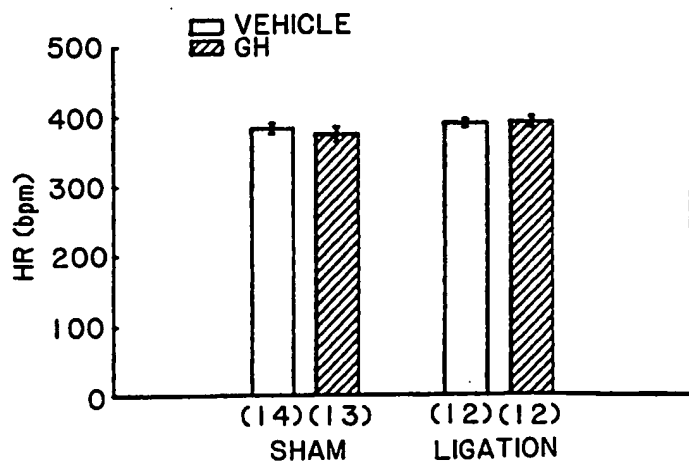


FIG. 3c

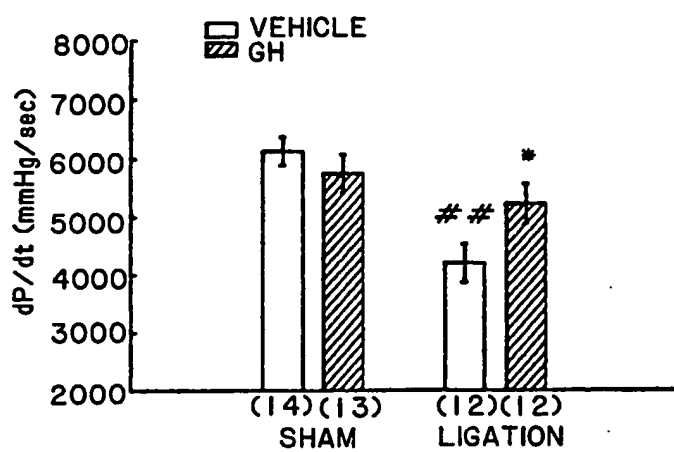


FIG. 4a

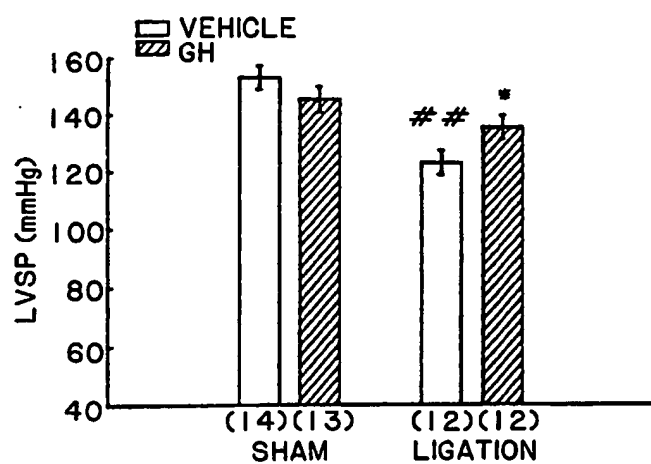


FIG. 4b

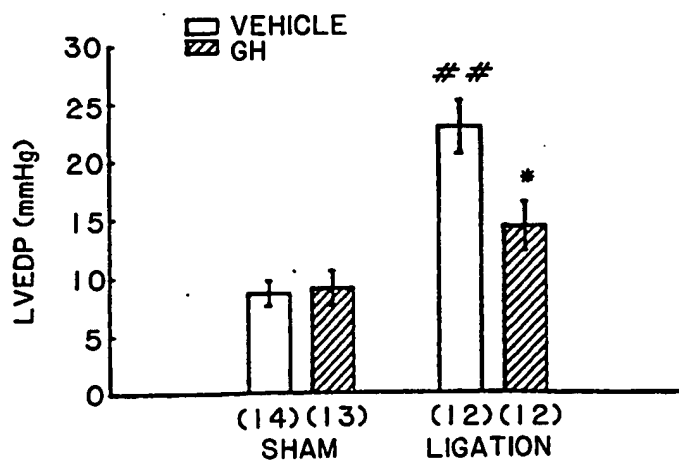


FIG. 4c

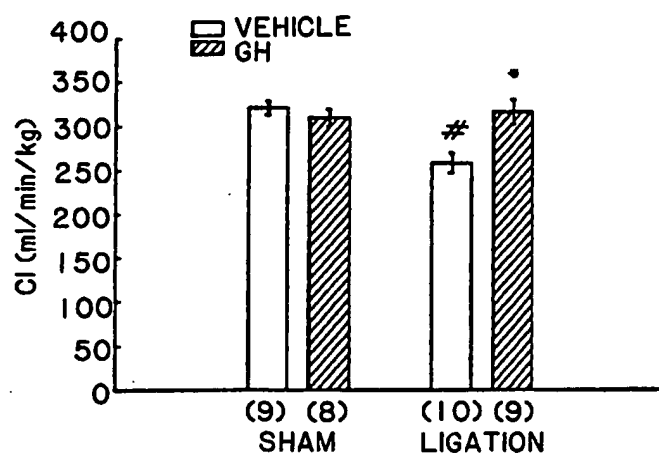


FIG. 5a

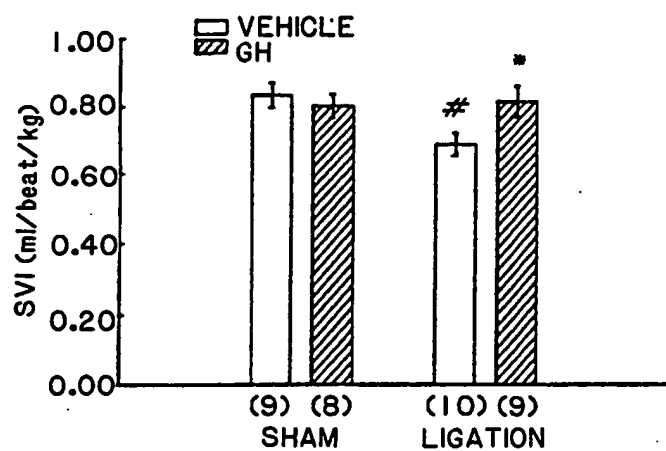


FIG. 5b

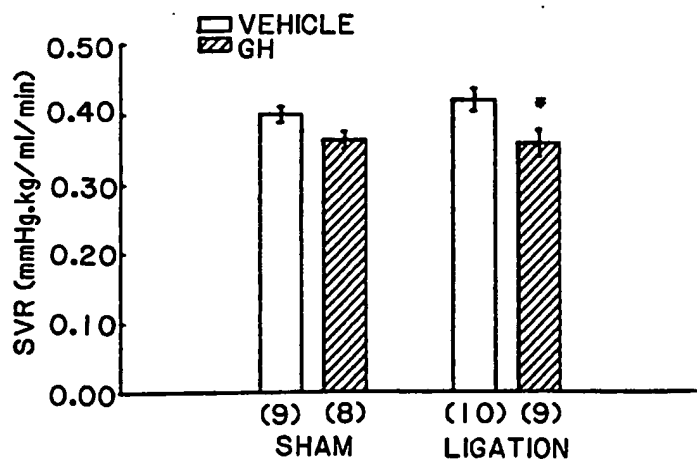


FIG. 5c

INTERNATIONAL SEARCH REPORT

Int. Application No
PCT/US 95/03760

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/27

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>THE LANCET, vol. 1, no. 8642, 15 April 1989 LONDON, GB, pages 838-839, R.C. CUNEO ET AL. 'CARDIAC FAILURE RESPONDING TO GROWTH HORMONE.' cited in the application see the whole document</p> <p style="text-align: center;">--- -/--</p>	1-6

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

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- "&" document member of the same patent family

Date of the actual completion of the international search

18 July 1995

Date of mailing of the international search report

16.08.95

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Authorized officer

Ryckebosch, A

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/03760

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHEMICAL ABSTRACTS, vol. 109, no. 5, 1 August 1988 Columbus, Ohio, US; abstract no. 32390n, L. THUESEN ET AL. 'INCREASED MYOCARDIAL CONTRACTILITY FOLLOWING GROWTH HORMONE ADMINISTRATION IN NORMAL MAN. AN ECHOCARDIOGRAPHIC STUDY.' page 97; cited in the application see abstract & DMB, DAN. MED. BULL., vol. 35, no. 2, 1988 pages 193-196, ----	1-6
A	WO,A,92 07578 (GENENTECH, INC.) 14 May 1992 see page 4, line 23 - line 31; claims 1,2 see page 9, line 5 - line 12 ----	1-6
P,X	CARDIOVASCULAR DRUGS AND THERAPY, vol. 9, no. 1, 1995 BOSTON, US, pages 125-131, R. YANG ET AL. 'EFFECTS OF GROWTH HORMONE IN RATS WITH POSTINFARCTION LEFT VENTRICULAR DYSFUNCTION.' see the whole document ----	1-6
O,P, X	CLINICAL RESEARCH (ABSTRACTS OF THE MEETING OF THE AMERICAN FEDERATION FOR CLINICAL RESEARCH, BALTIMORE US, APRIL 29-MAY 2, 1994), vol. 42, no. 2, April 1994 page 325A see the whole document -----	1-6

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/03760

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9207578	14-05-92	AU-A- 8941291	26-05-92
		CA-A- 2092718	26-04-92
		EP-A- 0554381	11-08-93
		JP-T- 6503320	14-04-94
		US-A- 5370870	06-12-94
